Expression and characterization of a dopamine D4R variant associated with delusional disorder

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Abstract Multiple genetic polymorphisms of the human dopamine D4 receptor (hD4R) have been identified including a 12 bp repeat in exon 1 associated with a psychotic condition called delusional disorder. Competition binding assays revealed minor pharmacological differences between the recombinant A1 (normal) and A2 (delusional) proteins with respect to quinpirole and the antipsychotic clozapine, however no functional differences were detected for receptor activation by dopamine, epinephrine, or norepinephrine. Our results suggest that this polymorphism may only confer susceptibility to delusional disorder in combination with other genetic or environmental factors.

Key words: DFR1; Polymorphism; Schizophrenia; Antipsychotic; Antidepressant

1. Introduction

Disturbances in dopaminergic pathways have been implicated in the etiology of several psychiatric disorders, and brain dopamine receptors are a key target for drugs used in the treatment of psychosis. Dopamine acts through five distinct dopamine receptor subtypes, D1–D5, which have been cloned and characterized. Among them, the dopamine D4 receptor (D4R) gene has received considerable attention in light of its anatomical, pharmacological, and structural features (reviewed in [1]). Moreover, among the dopamine receptor genes, D4R shows a uniquely high degree of genetic variation in the human population, with a 2–10-fold repeated region of 48 bp in the third exon of its coding sequence. Other known D4R gene polymorphisms include a polymorphic 12 bp repeat in exon 1 [2], a T′G coding transition at nucleotide 581 [3], and the presence of a nonsense 13 bp deletion in exon 1 [4]. More recently, two novel polymorphisms and a rare deletion variant have been reported [5].

The 12 bp repeat in exon 1 encodes a sequence of four amino acids in the extracellular N-terminal part of the receptor, which borders the first putative transmembrane domain. This sequence occurs as a two-fold repeat in the more common variant (A1 allele) and is represented only once in the rarer one (A2 allele). Previous results have suggested the involvement of this genetic variation in conferring susceptibility to delusional disorder [2]. Delusional disorder shows some of the same symptoms as schizophrenia, but differs in its course and pharmacological response [6,7]. Interestingly, patients suffering from delusional disorder respond poorly to neuroleptics [8,9], suggesting that this deletion may affect the pharmacological properties of D4R. These data prompted us to investigate possible functional consequences of this particular variant of the receptor.

2. Materials and methods

2.1. Materials

(−)Epinephrine, dopamine, haloperidol, (+)butaclamol, clozapine, lisuride, quinpirole, 7-OH-DPAT, sulpiride, loxapine, risperidone, thioridazine, chlorpromazine, doxepine, mianserine, 8-OH-DPAT, DOI and D-cycloserine were purchased from Research Biochemicals International (RBI). Norepinephrine and NMDA were obtained from Sigma, and 5-HT from Fluka. Raclopride was the kind gift of Nina Mohell, Astra (Sweden). Bromocryptine, pimozide, clomipramine, clonidine, fluvoxamine, fluoxetine, DZP, DMCM, L-DOPA, L745870, and Ro 15-4513 were obtained from the Roche chemical library. [^{3}H]Spiperone (89 Ci/mmol) and [^{35}S]GTPγS (1000 Ci/mmol) were purchased from Amersham. G418 and penicillin/streptomycin (10 000 units/10 000 μg, PS) were purchased from Gibco BRL, and fetal calf serum (FCS) was obtained from Amimed.

2.2. D4R variant expression constructs

The cDNA for the previously reported human D4.4R A1 allele [10] was subcloned into the *Hin*dIII/*Xba*I sites of pcDNA3 to create the expression plasmid pcDNA3-hD4.4-A1. A PCR fragment containing the entire exon 1 was generated from genomic DNA obtained from an individual homozygous for the 12 bp deletion using two primers bearing *Bam*H1 plus *Eco*RI and *Hin*dIII plus *Eco*RI restriction sites, respectively. This fragment was digested with *Hin*dIII/*Ear*I and ligated to the 1050 bp *Ear*I/*Xba*I fragment of pcDNA3-hD4.4-A1 to reconstruct the D4R A2 allele, which was subcloned into the *Hin*dIII/*Xba*I sites of pcDNA3 to create the pcDNA3-hD4.4-A2 expression plasmid. Expression plasmids were sequenced on both strands using the ABI Prism Taq DyeDeoxy Terminator Cycle Sequencing Kit and a Model 310A Genetic Analyzer from Applied Biosystems, and analyzed using the Sequence Analysis Software and Sequence Navigator (Applied Biosystems).

2.3. Production of stable cell lines

CHO-K1 cells were grown to 80% confluence in a six well Nunc plate, washed, and incubated for 30 min at 37°C in Optimem-Glutamax medium (Gibco). 2 μg of plasmid DNA (pcDNA3-hD4.4-A1 or pcDNA3-hD4.4-A2) and 12 μl lipofectamine (Gibco) were added to a total of 200 μl Optimem medium and incubated at room temperature for 30 min, then added dropwise to the cells. Six hours later cells were trypsinized, suspended in 100 ml selection medium (DMEM-F12, 1% PS, 5% FCS, 1 mg/ml G418), and plated into 100 mm dishes. Twelve

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Abbreviations: hD4R, human dopamine D4 receptor; DA, dopamine; DZP, diazepam; DMCM, ethyl 4-ethyl-6,7-dimethoxy-[9*H*]-pyrido[3,4-*b*]indole-3-carboxylate; CHO, Chinese hamster ovary cells

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days later cell colonies were transferred to 24 well plates. Cell clones were analyzed for surface expression of D4R protein using flow cytometry with the DFR1 monoclonal antibody as described in [11]. Positive clones were expanded and D4R expression was confirmed by saturation binding with [^{3}H]spiperone. Two cell clones, CHO-hD4.4-A1 and CHO-hD4.4-A2, were chosen for detailed pharmacological profiling.

2.4. Immunoblotting

CHO cells were transfected with pcDNA3-hD4.4-A1 or pcDNA3-hD4.4-A2 plasmids using lipofectamine as described above, and crude membrane fractions were prepared as described in [11]. Proteins were separated by 10% SDS-PAGE with a 4% stacking gel, transferred to nitrocellulose membrane, and immunoblotted with DFR1 antibody (1 μg/ml) at 4°C for 18 h. Labeled proteins were detected using horseradish peroxidase goat anti-mouse IgG (H+L) (Chemicon, 1:3000 dilution) with enhanced chemiluminescence according to manufacturer's instructions (ECL, Amersham). Protein content was determined by the BCA assay method.

2.5. Binding assays

CHO-hD4.4-A1 and CHO-hD4.4-A2 stable cell lines were grown to 80% confluence and harvested using 0.02% EDTA in PBS. Cell membranes were prepared and binding assays performed as described in [12].

2.6. [^{35}S]GTPγS binding assay

[^{35}S]GTPγS binding assays were performed as described in [12]. B_{max} values were determined directly for the GTPγS membrane preparations, and are noted in Section 3.

3. Results

3.1. Expression of D4R A1 and A2 allele variants

The cDNA encoding the full-length D4.4R [10], referred to as the A1 allele, was subcloned into the pcDNA3 expression vector (pcDNA3-hD4.4-A1). The D4R isoform carrying four copies of the putative third cytoplasmic loop repeat (called D4.4) was chosen, since this is the most abundant in the human population [13]. In order to construct the A2 variant, an entire exon 1 fragment was generated by PCR from genomic DNA obtained from an individual homozygous for the 12 bp deletion. This fragment was used to replace the corresponding sequence in pcDNA3-hD4.4-A1 resulting in a second expression construct, pcDNA3-hD4.4-A2.

CHO cells were transiently transfected with pcDNA3-hD4.4-A1 and pcDNA3-hD4.4-A2 plasmids, and crude membranes were analyzed for D4R expression by immunoblot analysis with the monoclonal antibody DFR1 [11]. A 47 kDa band was observed in both transfections confirming that the expression constructs encoded full-length D4R proteins (Fig. 1). The size of this band suggested that it did not contain glycosylation since it corresponded to the calculated molecular weight of the D4.4R polypeptide. No DFR1 immunoreactivity was observed in untransfected CHO cells, as expected. D4R aggregates observed at 90 and 130 kDa probably occur as a result of the high level of D4R protein expression since they were not observed in the stable CHO-D4.4 cell line reported previously [11].

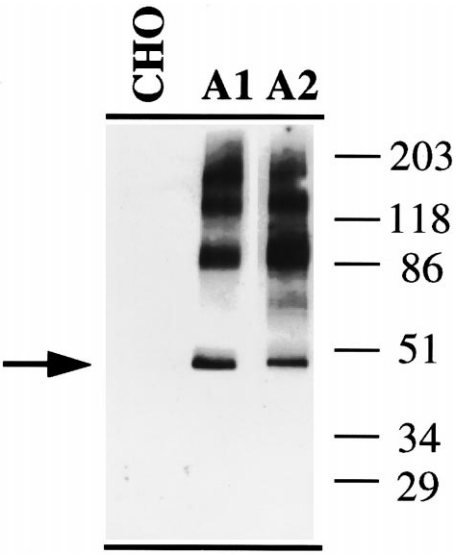

Fig. 1. Western blot analysis of A1 and A2 dopamine D4R variants with the monoclonal antibody DFR1. CHO cells were transiently transfected with pcDNA3-hD4.4-A1 and pcDNA3-hD4.4-A2 plasmids and crude membranes were separated by SDS-PAGE. A 47 kDa band corresponding to the D4R protein was labeled by DFR1 in both A1 and A2 membrane preparations, and no labeling was detected in untransfected CHO cells. Higher molecular weight D4R aggregates are also observed of approximately 95 and 150 kDa. 1 μg protein was loaded per lane, and film exposure time was 20 s.

CHO cells stably expressing the A1 and A2 D4R isoforms were obtained by transfection with pcDNA3-hD4.4-A1 and pcDNA3-hD4.4-A2 plasmids followed by selection in the presence of G418. A total of 24 clones of each variant were analyzed by flow cytometry with the monoclonal antibody DFR1 [11], from which four clones with the highest surface labeling were selected for expansion. Saturation binding analysis with [^{3}H]spiperone revealed two cell lines of interest, CHO-hD4.4-A1 and CHO-hD4.4-A2, which had B_{max} values of 0.56 ± 0.03 and 1.97 ± 0.17 pmol/mg protein, respectively. Scatchard analysis of [^{3}H]spiperone binding revealed binding constants (K_D values) of 371 ± 30 pM and 357 ± 40 pM ($n = 3$) for A1 and A2 variants, respectively (Fig. 2). This is in good agreement with published values for [^{3}H]spiperone binding to the D4.4R in CHO cells [12], and demonstrated no difference in affinity for spiperone between the A1 and A2 D4R variants.

Table 1
Inhibition constants (K_i values) of catecholamines at hD4.4R A1 and A2 variants

	hD4.4-A1			hD4.4-A2		
	High affinity		Low affinity	High affinity		Low affinity
	K_i (nM)	% of total[a]	K_i (nM)	K_i (nM)	% of total[a]	K_i (nM)
Dopamine	0.7 ± 0.3	73	35 ± 13	1.2 ± 0.4	69	44 ± 23
Epinephrine	8.4 ± 2.1	76	430 ± 10	13.5 ± 4.9	70	276 ± 143
Norepinephrine	35.1 ± 7.5	80	2200 ± 894	36.6 ± 6.9	71	1423 ± 258

Data are given as mean ± S.E.M. of three experiments. Competition of [^{3}H]spiperone (200 pM) was performed with 20 increasing concentrations of ligand ranging from 10^{-12} to 10^{-4} M. Two-site fits were calculated using LIGAND for the Macintosh.
[a]Percentage of high affinity sites.

3.2. Comparison of pharmacological profiles

It has recently been reported that the D4R receptor can be functionally activated by three different catecholamines: dopamine (DA), epinephrine (EP), and norepinephrine (NE) [12,14]. In competition binding experiments with [^{3}H]spiperone, inhibition constants (K_i values) for the high affinity binding sites of DA, EP, and NE were found to be 0.7, 8.4, and 35 nM, respectively in CHO-hD4.4-A1 crude membranes, consistent with previously reported values [12]. In each case, high affinity sites represented about 75% of the total binding sites. No significant differences were detected between the hD4.4-A1 and D4.4-A2 membranes with respect to K_i values or percentage of high affinity binding sites, which varied from 70 to 80% in each case (Table 1).

The pharmacological profiles of CHO-hD4.4-A1 and CHO-hD4.4-A2 membranes were further extended to include other dopaminergic compounds, as well as selected antipsychotic and antidepressant drugs. Quinpirole, a dopaminergic agonist, showed approximately two-fold lower affinity at the A2 variant, but no differences in K_i values were detected between the A1 and A2 variants for apomorphine, lisuride, 7-OH-DPAT, bromocryptine, butaclamol or L745870, a new D4R specific antagonist [15] (Table 2). Raclopride and sulpiride showed very low affinity for both A1 and A2 isoforms.

Table 2
Pharmacological profile of A1 and A2 recombinant D4.4R variants

	K_i (nM)	
	A1	A2
Dopaminergic agonists		
(−)Apomorphine	0.58 ± 0.09	0.67 ± 0.01
Lisuride	2.6 ± 0.2	2.2 ± 0.2
Quinpirole	4.2 ± 0.7	8.0 ± 1.6
7-OH-DPAT	39.5 ± 1.9	47.4 ± 3.9
Bromocryptine	130 ± 41	138 ± 52
Dopaminergic antagonists		
L745870	0.78 ± 0.03	0.60 ± 0.08
Butaclamol	107 ± 8	76.8 ± 1.4
Raclopride	4450 ± 330	3765 ± 475
Antipsychotics		
Haloperidol	3.3 ± 0.3	3.4 ± 1.5
Loxapine	7.2 ± 1.0	7.8 ± 1.0
Risperidone	7.4 ± 1.8	5.5 ± 0.7
Thioridazine	8.7 ± 2.0	7.3 ± 0.2
Chlorpromazine	11.0 ± 0.7	7.8 ± 0.5
Pimozide	12.6 ± 2.0	9.5 ± 0.5
Clozapine	25.5 ± 2.3	17.0 ± 0.2
Sulpiride	862 ± 114	1100 ± 403
Antidepressants		
Chlorimipramine	97.1 ± 5.7	90.6 ± 4.3
Doxepine	275 ± 17	260 ± 19
Mianserine	488 ± 93	370 ± 40
Fluvoxamine	> 1e−5	> 1e−5
Fluoxetine	> 1e−5	> 1e−5
Other		
5-HT	3710	2050
8-OH-DPAT	8110	9890
Clonidine	> 1e−5	> 1e−5
DOI	> 1e−5	> 1e−5
NMDA	> 1e−4	> 1e−4
DZP	> 1e−4	> 1e−4
DMCM	> 1e−4	> 1e−4
Ro 15-4513	> 1e−4	> 1e−4
D-Cycloserine	> 1e−4	> 1e−4
L-DOPA	> 1e−4	> 1e−4

Data are given as mean ± S.E.M of three experiments. Competition of [^{3}H]spiperone (200 pM) was performed with 20 increasing concentrations of ligand ranging from 10^{-12} M to 10^{-4} M.
DOI, 4-iodo-2,5-dimethoxyphenylisopropylamine; NMDA, *N*-methyl-D-aspartate; DZP, diazepam; DMCM, ethyl 4-ethyl-6,7-dimethoxy-9*H*-pyrido[3,4-*b*]indole-3-carboxylate.

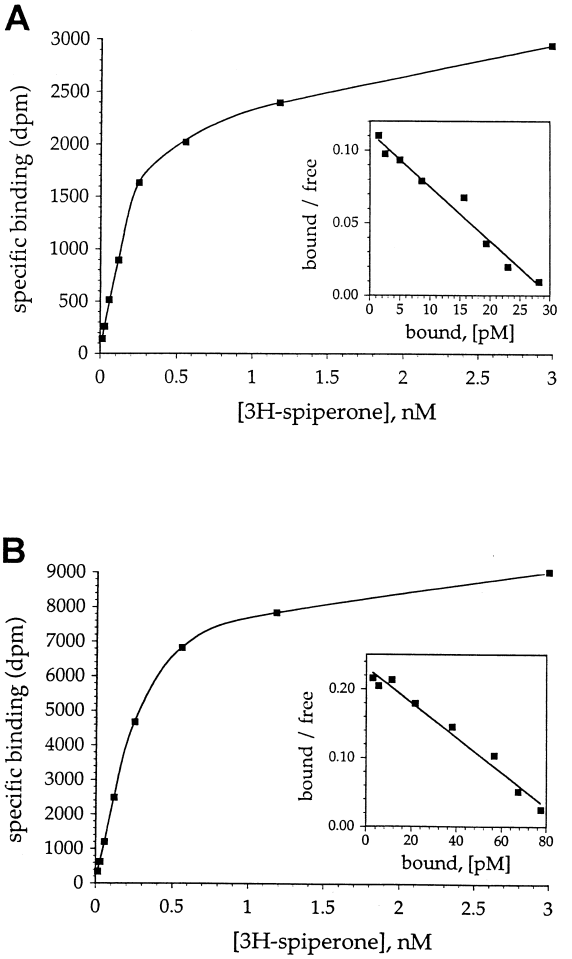

Fig. 2. Saturation binding and Scatchard transformation of [^{3}H]spiperone binding to A1 and A2 D4R variants expressed in CHO cells. CHO-hD4.4-A1 and CHO-hD4.4-A2 cell membranes (30–35 μg/assay) were incubated with [^{3}H]spiperone at 0.01–3 nM ($n = 3$) for 90 min at RT in the dark. Non-specific binding was determined in the presence of 10 μM (+)butaclamol. Scatchard analysis was performed using EBDA for Macintosh, and representative curves are shown. K_D values of 371 ± 30 pM and 357 ± 40 pM ($n = 3$) were determined for A1 and A2 D4R variants, respectively.

Eight antipsychotic compounds were tested including haloperidol, which showed approximately 3 nM affinity for both A1 and A2 variants. Clozapine showed a slight preference for the A1 allele, however there was less than two-fold difference in affinity compared to the A2 allele (25.5 ± 2.4 vs. 17 ± 0.2 nM for A1 and A2, respectively). Loxapine, risperidone, thioridazine, chlorpromazine, and pimozide all showed high affinity binding to the D4R, but no differences in K_i values between the A1 and A2 variants were identified.

Among the five antidepressant compounds tested, only clomipramine bound with appreciable affinity to the D4R showing similar K_i values of 97 and 91 nM for A1 and A2 variants, respectively. Doxepine and mianserine showed weak affinity for the D4R, with K_i values of 275 ± 17 and 488 ± 93 nM at

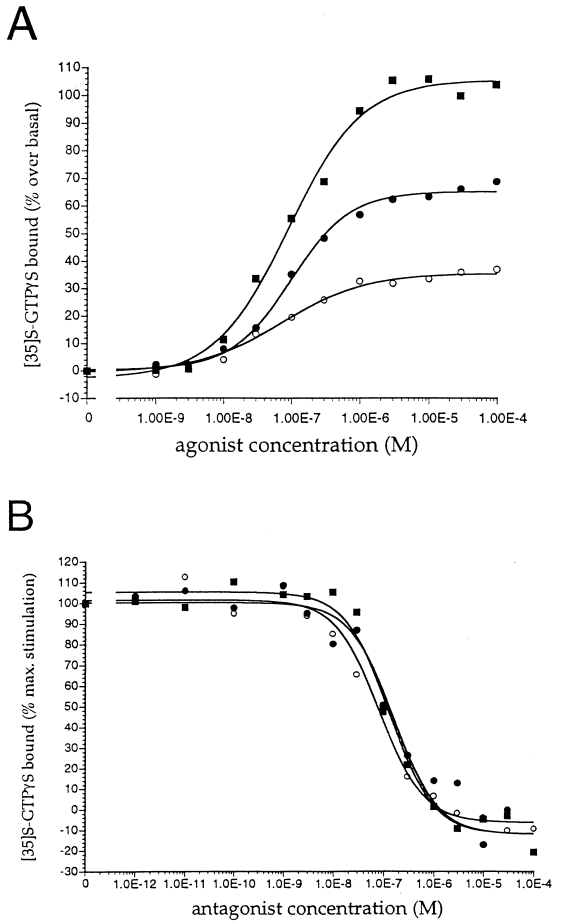

Fig. 3. Evaluation of A1 and A2 D4R functional properties as measured by [^{35}S]GTPγS binding assays. A: CHO-hD4.4-A1 (filled circles), CHO-hD4.4-A2 (filled squares), and CHO-hD4.4-A2-low (open circles) cell membranes were incubated with increasing concentrations of dopamine. Maximal receptor activation at the 100% level corresponded to 25 000 specific dpm. EC_{50} values of 91 ± 4, 95 ± 6, and 80 ± 5 nM (n = 3) were determined for A1, A2, and A2-low membranes, respectively. B: DA-induced [^{35}S]GTPγS binding in CHO-hD4.4-A1 (filled circles), CHO-hD4.4-A2 (filled squares), and CHO-hD4.4-A2-low (open circles) cell membranes is inhibited with similar potency by the neuroleptic haloperidol (IC_{50} values 101 ± 18, 91 ± 11, and 115 ± 45 nM, respectively).

the A1 allele, respectively, and no detectable differences detected between the A1 and A2 variants. Fluvoxamine and fluoxetine showed no interaction with either D4R isoform. Other compounds with little or no detectable affinity for either isoform included clonidine, D-cycloserine, L-DOPA, 5-HT, NMDA, and the serotonergic compounds DOI and 8-OH-DPAT. The benzodiazepines diazepam (DZP) and DMCM did not interact with either D4R variant, nor did Ro 15-4513, a weak inverse agonist which binds the diazepam-insensitive α6 subunit of the $GABA_A$ receptor.

3.3. Functional characterization

We have used an [^{35}S]GTPγS binding assay to compare hD4R activation in membranes prepared from CHO-hD4.4-A1 and CHO-hD4.4-A2 cells. In this assay the degree of stimulation is proportional to the level of receptor expression, as shown in Fig. 3A. DA-induced stimulation of [^{35}S]GTPγS binding reached 95% of basal levels in CHO-hD4.4-A2 membranes, where D4R expression levels were exactly 2.0 pmol/mg protein. CHO-hD4.4-A1 membranes, with a D4R expression level of 0.65 pmol/mg protein, had DA-induced stimulation levels of 58%. Despite the difference in expression levels, nearly identical EC_{50} values of 91 ± 4 and 95 ± 6 nM (n = 3) were determined for CHO-hD4.4-A1 and CHO-hD4.4-A2 membranes, respectively. To confirm that the higher A2 expression level was not concealing a lower agonist potency, a second cell clone expressing lower levels of the A2 isoform (CHO-hD4.4-A2-low) was also characterized. CHO-hD4.4-A2-low membranes had a receptor expression level of 0.34 pmol/mg protein with 32% stimulation over basal, and showed an EC_{50} value for DA of 80 ± 5 nM, consistent with that obtained with the CHO-hD4.4-A1 and CHO-hD4.4-A2 membranes. Thus, no significant difference between the A1 and A2 D4R variants with respect to potency of DA stimulation was detected.

In order to investigate possible differences in functional blockade of the D4R, antagonism of DA-induced [^{35}S]GTPγS binding by haloperidol (10^{-12}–10^{-4} M) was evaluated. IC_{50} values of 101 ± 18, 91 ± 11, and 115 ± 45 nM were determined for CHO-hD4.4-A1, CHO-hD4.4-A2, and CHO-hD4.4-A2-low membranes, respectively, demonstrating no significant difference between the A1 and A2 variants (Fig. 3B), as predicted from the similar K_i values obtained in competition binding assays.

4. Discussion

In stably transfected CHO cell lines expressing the hD4.4R, the pharmacological profiles of the A1 and A2 D4R variants were found to be nearly identical with respect to neurotransmitter binding (DA, EP, and NE) and affinity for the antipsychotic drugs tested including haloperidol, risperidone, and chlorpromazine. Minor differences were observed for clozapine, which showed almost two-fold higher binding affinity for the A2 variant, while the dopaminergic agonist quinpirole had slightly higher affinity for the A1 variant. However, functional activation of A1 and A2 D4R variants by dopamine, and antagonism of this response by haloperidol, were not significantly different in an in vitro [^{35}S]GTPγS binding assay.

Three adrenergic antagonists – yohimbine, prazosin, and propranolol – have previously been shown to be inactive at the D4R [12], despite the ability of EP and NE to act as potent agonists at this receptor. In this study we show that clonidine, another α receptor antagonist, also shows no affinity to the D4R. Antidepressant drugs tested here included the selective serotonin uptake inhibitors fluoxetine (Prozac) and fluvoxamine, which were inactive at the D4R as expected. Mianserine, a tetracyclic compound, showed low affinity to the D4R, while the two tricyclics doxepine and clomipramine showed significant receptor affinity. Clomipramine has also been reported to show moderate affinity to the D2R in vitro [16].

The lack of significant differences between A1 and A2 variants suggests that the N-terminal region of the D4R is not directly involved in receptor activation. This is consistent with the finding that DFR1, a monoclonal antibody raised against the extreme N-terminus of the D4R, did not alter DA-induced receptor activation or antagonism by haloperidol [11]. In immunoblotting experiments, we have found that both A1 and A2 D4R variants are labeled by DFR1, which was expected

since the DFR1 epitope does not overlap with the exon 1 polymorphic region.

Our results suggest that the difference in response to neuroleptic drug treatment observed in delusional disorder patients may not be a direct consequence of the 12 bp polymorphism in exon 1, however the functional relevance of the A2 allele in a complex disorder such as psychosis may only be detected when expressed in the appropriate genetic background. Further experiments should explore the pharmacology of A1 and A2 isoforms when these sequences are combined with other D4R polymorphismic variants, for instance D4.7-A1 vs. D4.7-A2, or D4.2-A1 vs. D4.2-A2 alleles. Investigation of receptor-mediated cAMP accumulation and sodium sensitivity of ligand binding, paradigms in which the D4.7 variant shows somewhat altered responses [10,17], might also be used to reveal subtle differences. Importantly, additional studies of patient populations will be required to understand how this variation, perhaps in combination with other genetic and environmental factors, may confer susceptibility to delusional disorder.

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